

# *N*-Acetyltaurine as a novel urinary ethanol marker in a drinking study

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Received: 11 May 2016 / Revised: 7 July 2016 / Accepted: 1 August 2016  
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**Abstract** The forensic utility of *N*-acetyltaurine (NAcT) in urine as a marker for ethanol intake was examined. A HILIC-based liquid chromatography method for the mass spectrometric determination of NAcT, taurine, and creatinine in urine was developed and validated to investigate NAcT formation and elimination in a drinking study. Thereby, eight subjects ingested 0.66 to 0.84 g/kg alcohol to reach a blood alcohol concentration (BAC) of 0.8 g/kg. Blood and urine were taken every 1.5–2 h, during the first 8 h. NAcT and taurine levels were measured and corrected for the urine's dilution by normalization to a creatinine concentration of 1 mg/mL. For the determination of NAcT and taurine, uncorrected lower limits of quantitation (LLOQs) were at 0.05 µg/mL of urine. In the drinking study, NAcT proved to be an endogenous compound, which is present at a range of 1.0 to 2.3 µg/mL in urine of alcohol-abstinent subjects. Maximum NAcT concentrations were reached in samples taken 3 to 6 h after the start of drinking, whereby an upregulation in *N*-acetyltaurine could be found for all the subjects. The mean peak concentrations ( $\bar{c}_{\max}$ ) of  $14 \pm 2.6$  µg/mL (range 9–17.5 µg/mL) were reached. Within 24 h, the NAcT levels

declined to endogenous concentrations. The detectability of NAcT was found to be slightly shifted compared to BAC: When BAC was not detectable anymore, NAcT levels were still elevated. After 24 h, when ethyl glucuronide (EtG) and ethyl sulfate (EtS) were still detectable, NAcT concentrations showed endogenous levels again. Positive NAcT results can be used as an indicator for recent alcohol consumption. A direct relationship between NAcT and taurine concentrations could not be found.

**Keywords** *N*-Acetyltaurine · Taurine · Alcohol marker · Abstinence monitoring · Urine analysis · LC-MS/MS

## Introduction

Alcohol abuse and its medical and social effects pose a major challenge in today's society. Of all the deaths, 5.9 % are directly linked to excessive alcohol consumption [1]. In order to prove alcohol abstinence, various direct alcohol markers are currently analyzed in blood, urine, and hair, such as ethyl glucuronide (EtG), ethyl sulfate (EtS), and phosphatidylethanol (PEth) [2]. *N*-Acetyltaurine (NAcT) was found to be an ethanol-related direct marker in mice and is generally not included in routine analysis [3]. The suitability and detectability of NAcT as a forensic marker in human being have not been investigated so far [4]. The formation of NAcT requires taurine, acetyl-CoA, and/or acetate as a substrate [3]. As acetate and acetyl-CoA are closely related to ethanol metabolism, NAcT levels increase after alcohol consumption [5]. An increase of NAcT has also been observed after an endurance exercise, such as a marathon, mostly due to the ketogenic state of the body. During this period, acetate is produced, which serves as an acetyl donor for the formation of NAcT from taurine. The concentration of NAcT after such an

**Electronic supplementary material** The online version of this article (doi:10.1007/s00216-016-9855-7) contains supplementary material, which is available to authorized users.

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endurance exercise was reported to be around 2.8 µg/mL [6]. In this paper, we provide detailed profiles of NAcT concentrations in human urine during the course of a drinking study: Our results provide an overview of the endogenous NAcT concentrations of abstinent persons and its increase after the consumption of a predefined amount of alcohol.

## Materials and methods

### Reagents

Taurine, acetic anhydride, creatinine, and creatinine- $d_3$  were obtained from Sigma-Aldrich (St. Louis, USA). Ethanol, pyridine, acetic acid, and ammonium acetate were purchased from Merck (Darmstadt, Germany). Water was produced with a Milli-Q water system from Millipore (Billerica, USA). Taurine- $d_4$  was ordered from Toronto Research Chemicals (Toronto, Canada). Acetonitrile, p.a., was obtained from Acros Organics (Geel, Belgium). Acetyltaurine was obtained from Carbosynth (Berkshire, UK). Deuterated DMSO (99.9 %) was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA).

### Synthesis of internal standard

Deuterated NAcT was synthesized by dissolving 2 mg of taurine- $d_4$  in 400 µL of water. Then, 143 µL pyridine and 115 µL acetic anhydride were added. The reaction mixture was stirred for 20 h at room temperature. The solvents were evaporated to dryness at 60 °C under vacuum (approximately 0.18 bar) with a CentriVap concentrator from Labconco® (Biolabo Scientific Instruments, Switzerland). The residue was reconstituted in 0.8 mL water and stored at −20 °C.

### Drinking study design

Eight volunteers (six men and two women, aged 19 to 26 years), who had been abstinent for at least 2 weeks, ingested a single dose of alcohol (66 proof/37.5 % vodka mixed with a soft drink), which led to an approximate blood alcohol concentration (BAC) of 0.8 g/kg. The alcohol doses (125–210 mL) were calculated for each person by the Widmark formula with individually adjusted reduction factors based on the weight and size of the individual [7]. Blank blood and urine samples were obtained from all subjects prior to the experiment. One hundred minutes after the start of drinking, the first blood and urine samples were taken. Three more samples were taken during the next 5 h. Further, urine samples were taken every 24 h after the experiment for 96 h. The presented procedure is based on the drinking study procedure for fatty acid ethyl ester determination [8].

### Determination of BAC

BAC was determined in lithium-heparinized blood by a validated headspace gas chromatography method with flame ionization detection (HS-GC-FID). According to Swiss forensic guidelines, the samples were analyzed with two different GC-FID systems with two measurements each, analogously to BAC determination in serum [9].

### Determination of EtG and EtS

EtG and EtS in urine were analyzed by a validated LC-MS/MS method. The method is used for routine analysis at the Institute of Forensic Medicine Bern for samples from withdrawal therapy to monitor abstinence [7].

### NMR spectroscopy

NMR data were collected on a Bruker Avance II (500 MHz;  $^1\text{H}$ ) spectrometer from Bruker BioSpin (Fällanden, Switzerland) equipped with a 1.7-mm triple-resonance ( $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{31}\text{P}$ ) micro-probe head. Approximately 0.5 mg of NAcT (used as received) was dissolved in 45 µL of DMSO- $d_6$  and transferred into 1.7-mm NMR tubes. All spectra were recorded and processed using the TopSpin® software (version 3.2, Bruker BioSpin). One-dimensional (1D)  $^1\text{H}$ -NMR experiments were recorded using the standard zg30 pulse program of the Bruker pulse sequence library. The spectral width was 12,000 Hz; a 2-µs pulse (30° tip angle) was applied; the acquisition time was 2.62 s, corresponding to 65,536 complex points; and the relaxation delay was 10 s to ensure quantitative conditions. The spectrum was recorded using 128 scans, for a total experimental time of approximately 25 min. All FID values were multiplied by an exponential function with a 0.3-Hz broadening line prior to Fourier transformation and zero-filled to 131,072 data points. Spectra were processed using automatic phasing, baseline correction and calibrated to the residual DMSO peak ( $\delta$  2.50 ppm). The purity of NAcT was estimated by comparing the integral of the  $\text{CH}_2\text{-SO}_3\text{H}$  protons of NAcT at  $\delta$  2.69 ppm with the integral of the protons of a  $\text{CH}_2$  group from an impurity at  $\delta$  2.88 ppm.

### Instrumentation for NAcT analysis

The liquid chromatography-mass spectrometry system consisted of an UltiMate® 3000 UHPLC+ focused system with an UltiMate® 3000 RS autosampler and a heated column compartment from Dionex (Olten, Switzerland) connected to a QTrap 5500 mass spectrometer from Sciex (Toronto, Canada). The whole system was controlled by Analyst 1.6.2 software. Chromatographic separation was performed with a SeQuant® ZIC®-HILIC 3.5-µm, 100 Å, 150 × 2.1 mm PEEK-coated HPLC column from Merck (Darmstadt,

**Table 1** Mass spectrometric parameters and retention time for *N*-acetyltaurine, taurine, and creatinine quantitation in urine

Compound	Q1 ( <i>m/z</i> )	Q3 ( <i>m/z</i> )	Time (ms)	DP (V)	CE (V)	CXP (V)	RT (min)
NAcT SRM1	166	107	20	−120	−30	−16	4.2
NAcT SRM2	166	124	20	−120	−30	−16	
NAcT- <i>d</i> <sub>4</sub>	170	128	20	−120	−30	−16	
Creatinine SRM1	112	68	20	−55	−27	−8	5.0
Creatinine SRM2	112	41	20	−55	−27	−8	
Creatinine- <i>d</i> <sub>3</sub>	115	42	20	−55	−27	−8	
Taurine SRM1	123.9	79.8	20	−5	−26	−21	4.9
Taurine SRM2	123.9	123.9	20	−5	−10	−16	
Taurine- <i>d</i> <sub>4</sub>	127.9	79.4	20	−105	−30	−19	

Germany), heated at 30 °C, with a flow rate of 0.3 mL/min. Mobile phase A consisted of a 5 mM ammonium acetate buffer at pH 5.72 with 0.1 % formic acid, and mobile phase B consisted of acetonitrile with 0.1 % formic acid. The analytes, depicted in Table 1, were separated with the following 14-min gradient: 0 to 1 min, 95 % B; 1 to 4.5 min, 95 to 20 % B linear; 4.5 to 9 min, 20 % B; 9 to 10 min, 20 to 95 % B linear; and 10 to 14 min, 95 % B. The mass spectrometer was operated in electrospray negative selected reaction monitoring (SRM) mode with an ion spray voltage of −4250 V and a source temperature of 550 °C, collision gas at 40, curtain gas at 35, gas 1 at 40, and gas 2 at 40. The resulting cycle time was 0.275 s.

### Determination of *N*-acetyltaurine, taurine, and creatinine

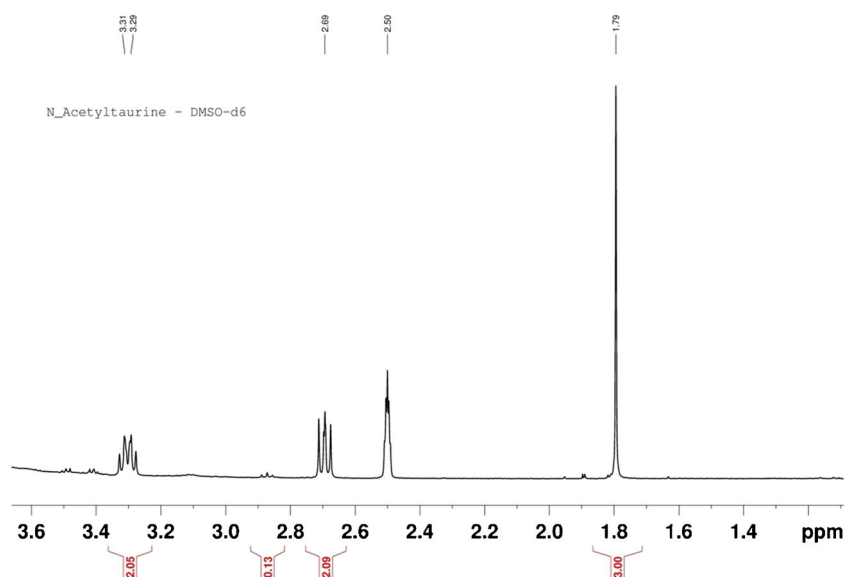
Urine samples were prepared by adding 50 µL of urine together with 150 µL acetonitrile (containing the internal standards) into a 2-mL Eppendorf tube and shaking at 1500 rpm on a VXR basic Vibrax (IKA, Staufen, Germany). Afterwards, the samples were centrifuged for 10 min at 16,000g and 100 µL of the supernatant was transferred into a 1.5-mL

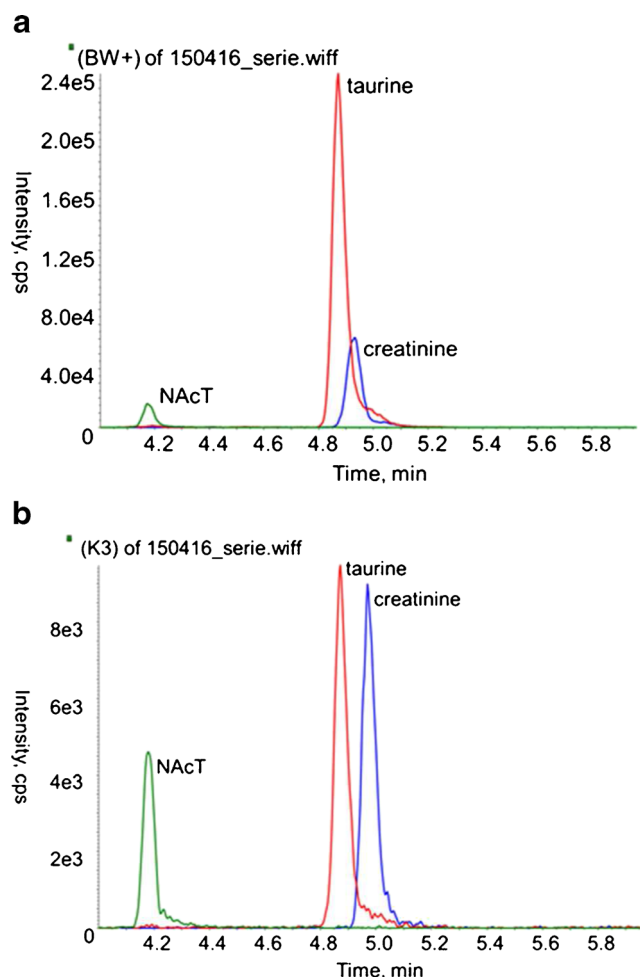
snap/crimp champagne glass vial. The organic phase was evaporated to dryness at 60 °C under vacuum (approximately 180 mbar) with a CentriVap concentrator from Labconco® (Biolabo Scientific Instruments, Switzerland). The residue was dissolved in 400 µL of reconstitution buffer (95 % acetonitrile and 5 % of 5 mM ammonium acetate buffer at pH 5.72, with 0.1 % formic acid). An aliquot of 10 µL was injected into the LC-MS/MS system. A blank sample (urine from an abstinent person without internal standard) and a zero sample (urine from an abstinent person with internal standard) were included in each series. NAcT and taurine concentrations were normalized to a creatinine value of 1 mg/mL in order to correct for the urine's dilution.

### NAcT method validation

Method validation for the determination of NAcT, taurine, and creatinine was performed according to FDA guidelines [10]. The presented procedure is based on a validation procedure previously used for fatty acid ethyl ester determination [8]. Selectivity, linearity, limit of quantification, imprecision (expressed as the relative standard deviation

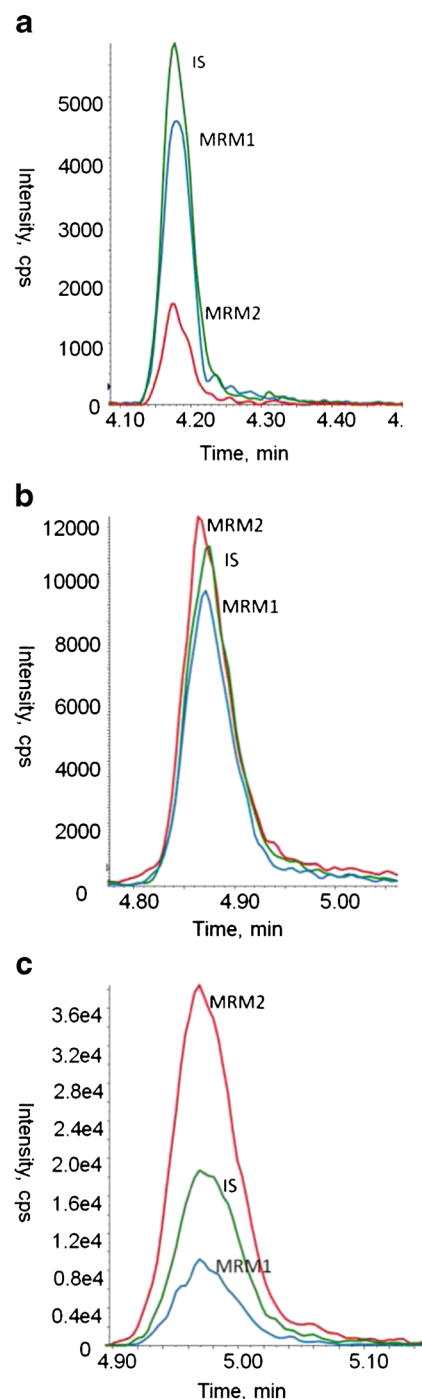
**Fig. 1** High field part of the 500 MHz <sup>1</sup>H-NMR spectrum of *N*-acetyltaurine dissolved in DMSO-*d*<sub>6</sub> recorded at 298 K. The resonances at δ 1.79, 2.69, and 3.30 ppm belong to *N*-acetyltaurine, while the resonance at δ 2.88 ppm belongs to impurity





**Fig. 2** Typical SRM1 chromatograms of a urine sample (a), with NAcT concentrations in the endogenous range, and a calibrator sample (b) with *N*-acetyltaurine 2.5  $\mu\text{g/mL}$ , taurine 2.5  $\mu\text{g/mL}$ , and creatinine 20  $\mu\text{g/mL}$

(RSD%), and accuracy (expressed as the mean relative error (RE%)) were determined. According to the guidelines, the mean value for accuracy and imprecision of quality control samples over six measurements, during three series, had to be within  $\pm 15\%$  of the actual value, except at the limit of quantification (LOQ), where a deviation of up to  $\pm 20\%$  is accepted. Selectivity was determined by testing six blank urine samples from alcohol-abstinent people (abstinence period of more than 2 weeks) for interferences of endogenous matrix components or metabolites, which could disturb the signals of the analytes or internal standards. Additionally, the feasibility of a standard addition-based method was investigated, by using urine from an abstinent subject for the calibration. For the final validation, working solutions containing NAcT, taurine, and creatinine for the calibration samples were prepared in water. Internal standard signal was used in order to correct for losses during sample preparation and ionization differences throughout the measurements. The area of the analyte signal was thereby divided by the



**Fig. 3** Detailed chromatograms for *N*-acetyltaurine 2.5  $\mu\text{g/mL}$  (a), taurine 2.5  $\mu\text{g/mL}$  (b), and creatinine 20  $\mu\text{g/mL}$  (c) with SRM1, SRM2, and internal standard (IS)

area of the internal standard signal. The following concentrations of NAcT and taurine were used to establish the calibration curve: 0.05, 0.1, 0.4, 2, 6, 12, and 50  $\mu\text{g/mL}$ . For creatinine, the following concentrations were used: 20, 100, 300, 600, and 2500  $\mu\text{g/mL}$ . All calibration curves were recorded twice on three different days. Precision and accuracy were determined by

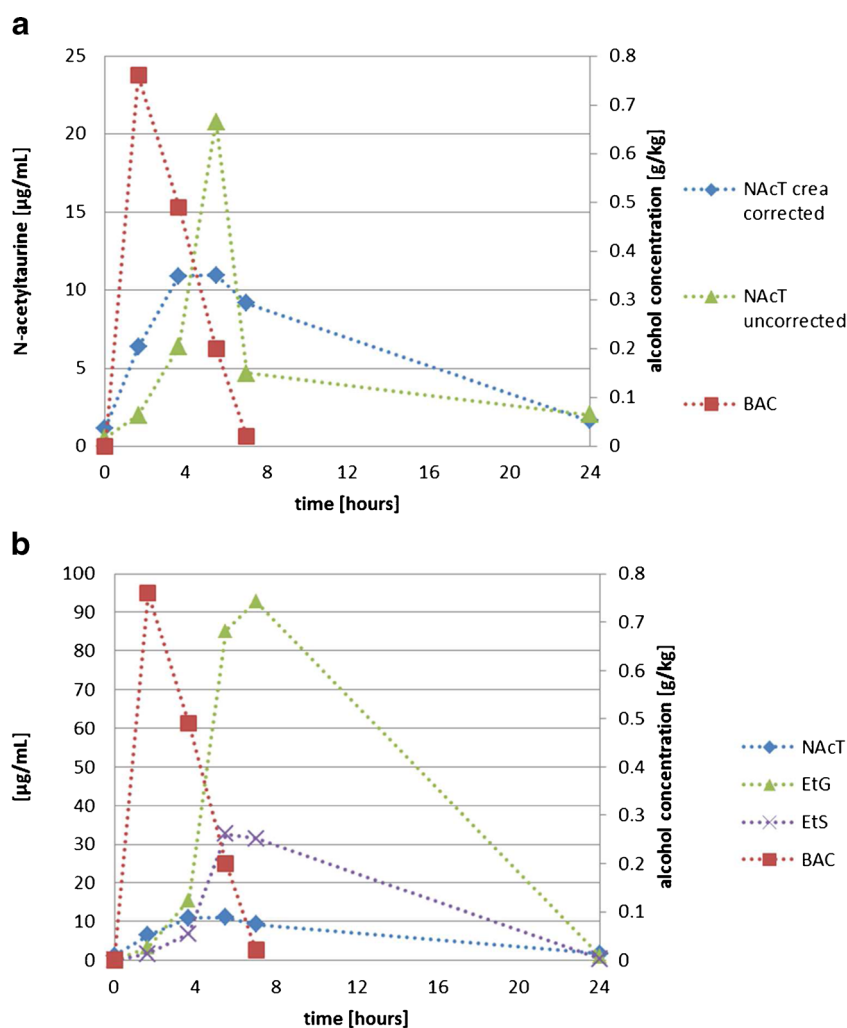
preparing quality control (QC) samples spiked at different NAcT and taurine concentration levels of 0.05, 0.5, 2.5, 5, and 32.5  $\mu\text{g/mL}$  and creatinine levels of 20, 100, 200, and 1300  $\mu\text{g/mL}$ . Carry-over was measured by injecting the highest calibrator three times, followed by a pure water sample to test if substances from the previous injection were carried over to the next measurement. Matrix effects, recovery, and extraction efficiency were analyzed by post-extraction addition in five different urine samples [11].

## Results and discussion

### $^1\text{H-NMR}$ analysis of *N*-acetyltaurine

The high field part of the  $^1\text{H-NMR}$  spectrum of NAcT dissolved in  $\text{DMSO-}d_6$  is shown in Fig. 1. The purity of NAcT was determined to be 94–95 % (see the “Material and methods” section).

**Fig. 4** (a) Comparison of *N*-acetyltaurine concentration with and without correction for the dilution of the urine. (b) Comparison of EtG, EtS, BAC, and NAcT in the same urine sample



### NAcT method validation

The calibration curve for determining the selectivity had to be measured in water, since the urine samples available contained at least one of the analytes investigated. Alternatively, the use of urine for establishing the calibration curve with incorporation of a correction for endogenous concentrations, based on a linear regression, would be possible. However, as this method does not allow quantifying below the concentration levels of the urine itself, it was disregarded. Extracted ion chromatograms for a blank urine specimen and a calibrator are shown in Fig. 2. The endogenous NAcT levels of 14 abstinent individuals proved to be close to each other (0.5–2.3  $\mu\text{g/mL}$ ). To analyze the linearity of the developed method, a linear calibration model with weighting  $1/x$  for NAcT and  $1/x^2$  for taurine and creatinine was used with spiked concentrations in the calibration range. Extracted ion chromatograms for SRM1, SRM2, and internal standards are shown in Fig. 3. For all calibration curves, the correlation coefficients ( $R^2$ ) from least squares regression were  $>0.9971$ , showing that the method is linear. The following LOQs were established: 0.05  $\mu\text{g/mL}$  for

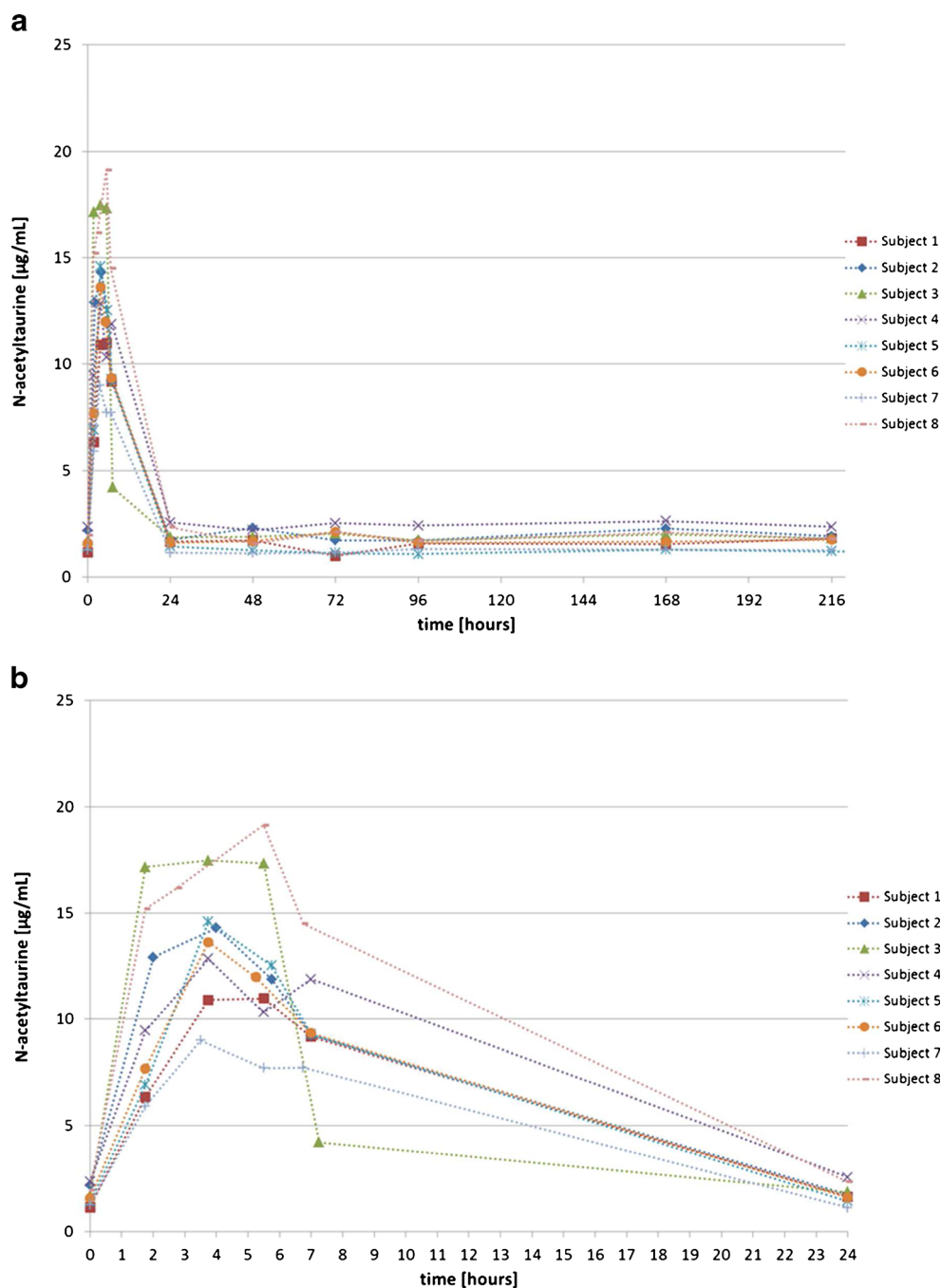


NAcT and taurine and 20 µg/mL for creatinine. The limit of detection (LOD) for NAcT and taurine was established by gradually diluting the solutions until the chromatogram displays a signal-to-noise ratio of 3:1. This was achieved at a concentration of 0.025 µg/mL. The LOD of creatinine was below 1 µg/mL. Imprecision and accuracy were in acceptable ranges: All QC samples had measured concentrations within  $\pm 15$  % of target, in accordance with FDA guidelines [10]. The mean intra-assay accuracy was 93–108 %, and the mean inter-assay accuracy was 93–105 % of the target (see [SS1](#)). The intra-assay imprecision was 0.7–14 %, and the inter-assay

imprecision was 0.6–4 % (see Electronic Supplementary Material (ESM), Table [S1](#)). The relative ion intensity for SRM2 was  $31 \pm 2$  % for NAcT,  $128 \pm 20$  % for taurine, and  $439 \pm 26$  % for creatinine, compared to the ion intensity of SRM1.

The carry-over for NAcT and taurine was below 0.1 %, and the carry-over for creatinine was at 0.2 %. NAcT showed an adequate extraction efficiency of  $43 \pm 9$  %, the recovery was  $35 \pm 5$  %, and the matrix effects were  $83 \pm 7$  %. For taurine, an adequate extraction efficiency of  $70 \pm 22$  % was obtained, the recovery was  $43 \pm 16$  %, and the matrix effects were 62

**Fig. 5** Time course for *N*-acetyltaurine for all eight subjects. An overview of *N*-acetyltaurine over 9 days can be seen in (a). The first 24 h is depicted in (b)



$\pm 13\%$ . Finally, creatinine demonstrated an adequate extraction efficiency of  $55 \pm 13\%$ , the recovery was  $53 \pm 11\%$ , and the matrix effects were  $96 \pm 12\%$ . Extracted quality control samples in the range of sample concentrations (0.5, 5, and  $32.5\ \mu\text{g/mL}$ ) were stable up to 72 h in the autosampler, after three freeze thaw cycles at about  $-18\ ^\circ\text{C}$ , and after storage for 7 days at  $4\ ^\circ\text{C}$ .

### Drinking study

The group of participating volunteers was very homogenous with respect to age and body mass index (BMI) with a mean age of  $23.5 \pm 2.4$  years (range 19–26 years) and a BMI of  $21.3 \pm 2.5\ \text{kg/m}^2$  (range  $17.7\text{--}25.1\ \text{kg/m}^2$ ). All blood samples collected prior to the drinking test were negative for ethanol. Endogenous NAcT levels in urine were present at a concentration range of 1.0 to  $2.3\ \mu\text{g/mL}$ . Maximum BACs were reached 1.75 h after the start of drinking with a mean maximum BAC of  $0.76 \pm 0.06\ \text{g/kg}$  (range  $0.66\text{--}0.84\ \text{g/kg}$ ). After drinking the calculated amounts of vodka, mean maximum NAcT concentrations of  $14 \pm 2.6\ \mu\text{g/mL}$  (range  $9\text{--}17.5\ \mu\text{g/mL}$ ) were reached within 3 to 6 h. Figure 4 shows the time courses of subject 1 for corrected and uncorrected NAcT concentrations. Overall, the correction for urine dilution by normalization to creatinine ( $1\ \text{mg/mL}$ ) proved to be reasonable. Detailed time courses for NAcT and BAC concentrations in each subject can be found in [SS2](#). All subjects showed similar NAcT profiles, as clearly demonstrated in Fig. 5. The peak concentrations and the elimination curve of NAcT in urine were slightly shifted in time—compared to the blood alcohol concentration. After 3.5 h, the concentration of NAcT in urine started to decrease. Interestingly, the BAC reached zero after about 7 h, while NAcT concentrations were still elevated in urine. NAcT concentrations around the endogenous levels were observed in the samples taken 24 h after the start of drinking, while overnight (from 8 to 24 h after the start of drinking), no samples were collected. A comparison of EtG, EtS, and NAcT is shown in [SS2](#) for all subjects. Considering generation and elimination, the increase in EtG and EtS concentrations is clearly higher compared to NAcT, and the maximum peak concentration is shifted by about 2 to 4 h. After 24 h, EtG ( $1.95 \pm 1.5\ \mu\text{g/mL}$ ) and EtS ( $0.65 \pm 0.6\ \mu\text{g/mL}$ ) were still detectable at low concentrations, while after 48 h, EtG and EtS could not be detected anymore. In contrast to NAcT, the two established markers EtG and EtS have the advantage of being non-detectable when no alcohol was consumed. This simplifies the interpretation of EtG and EtS results significantly, as endogenous and alcohol consumption-related concentration changes are non-existing. The relationship between taurine and NAcT concentrations could not be found: NAcT concentrations are about  $33 \pm 98\%$  (range  $0.34\text{--}727\%$ ) of the concentration of taurine.

### Conclusion

The concentration of N-acetyltaurine, which can be detected as an endogenous compound, significantly increases in urine after ingestion of ethanol. Compared to BAC, NAcT exhibits a longer detection window, but 24 h after a single ethanol uptake leading to a BAC of  $0.8\ \text{g/kg}$ , NAcT concentrations have decreased to endogenous concentrations. So far, we did not find any indication that would indicate a prolonged elimination time for NAcT. The long-term consumption of ethanol and the respective window of NAcT detectability after the last consumption have not yet been investigated. The detection window of EtG and EtS in urine proved to be longer than that of NAcT, as low concentrations of these analytes were still detectable after 24 h. Further studies are necessary to investigate endogenous levels in abstinent people in order to establish a reference range in healthy subjects and to study NAcT formation and elimination after regular consumption of higher amounts of alcohol, in blood and urine. So far, the analysis of NAcT levels bears no advantage when compared to EtG or EtS analysis. However, it can serve as a further indication for alcohol consumption. Additionally, the study of patients with metabolic disorder, such as kidney insufficiency as a potential cause for delayed NAcT clearance, or metabolic acidosis, e.g., in diabetes, as a reason for increased blood acetate concentration and thus increased endogenous NAcT levels, may be investigated. Moreover, the effects of taurine metabolism disorder or taurine supplementation, e.g., in energy drinks, remain unknown.

**Acknowledgments** We would like to thank Sidonia Guggisberg, Anita Iannone, Anja Kaiser, Severine Krönert, Nadja Utiger, and Thomas Wüthrich of the Forensic Toxicology and Chemistry Laboratory of the Institute of Forensic Medicine Bern for the determination of blood alcohol concentrations.

**Compliance with ethical standards** The drinking study has been approved by the Cantonal Ethics Commission Bern (064/13) on March 3, 2014. The participants of the drinking study gave informed consent for the analysis of direct alcohol marker in the collected samples.

**Conflict of interest** The authors declare that they have no conflict of interest.

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